Novel Rapid Test for Detecting Carbapenemase

Yanfang Feng,[1] Akilan Palanisami,[1] Jerrin Kuriakose, Michael Pigula, Shoaib Ashraf, Tayyaba Hasan

Author affiliations: Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA (Y. Feng, A. Palanisami, J. Kuriakose, M. Pigula, S. Ashraf, T. Hasan); Harvard-MIT Health Sciences and Technology, Cambridge, Massachusetts, USA (T. Hasan)

We developed a carbapenemase test based on the ability of imipenem to inhibit noncarbapenemase β-lactamases. The test uses bacterial isolates with a fluorescent β-lactamase substrate, producing objective results with 100% sensitivity and specificity in 10 minutes. The assay is inexpensive and consists of only 1 mixing step.

As a potent β-lactamase, carbapenemase can degrade almost all β-lactam antimicrobial drugs, including the carbapenems, regarded as the last line of therapy for many life-threatening infections (*1,2*). Various epidemic types of carbapenemase have been reported globally, including *Klebsiella pneumoniae* carbapenemase, Verona integron-encoded metallo-β-lactamase, *Serratia marcescens* enzyme, imipenem-hydrolyzing β-lactamase, New Delhi metallo-β-lactamase, oxacillinase, metallo-β-lactamase, and São Paulo metallo-β-lactamase (*1*). If uncontrolled, the spread of these carbapenemases is expected to increase therapeutic failure and leave many patients with no effective treatment options.

Despite the urgency, timely carbapenemase detection remains a challenge for microbiology laboratories. Phenotypic assays are inexpensive and easily performed, but their use requires 24–48 hours and many lack sensitivity or specificity (*3*). The widespread use of other assays (e.g., molecular tests of carbapenemase genes, mass spectrometry detection of carbapenem hydrolysis) is impeded by the expertise required to perform them and their cost (*4,5*). The recently developed (2012) Carba NP test and variants are elegant solutions, but their use requires up to 2 hours (*6*). Further improvements in test rapidity and simplicity are highly desirable, especially for patients in critical condition, who need immediate therapy and infection control action.

We demonstrate that by using fluorescence identification of β-lactamase activity (FIBA), carbapenemase production in bacteria can be detected

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[1]These authors contributed equally to this article.

sensitively and specifically in 10 minutes, with only 1 step. FIBA uses a dark fluorescence probe, β-LEAF (β-lactamase enzyme–activated fluorophore), which turns fluorescent when cleaved by β-lactamases, including penicillinases, extended-spectrum β-lactamases (ESBL), AmpC β-lactamases, and carbapenemases (*7,8*). Thus, the rate of fluorescence increase (hereafter called increase rate) is a measure of the bacterial β-lactamase activity and is reduced as the β-lactamase activity is hampered. For a noncarbapenemase β-lactamase, the increase rate will be reduced by the addition of imipenem, which binds the enzyme active site and blocks β-LEAF access (*2*). In contrast, the increase rate for a carbapenemase is relatively unaffected by imipenem addition because carbapenemase is able to rapidly cleave the imipenem and relieve the inhibition (*1*). Accordingly, bacteria that produce carbapenemases can be detected by comparing the increase rate with and without imipenem (Figure; Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/18-1655-App1.pdf).

FIBA is performed in a 96-well plate. Each well contains 50 μL of 20 μmol/L β-LEAF, 25 μL of phosphate-buffered saline with or without 40 μmol/L imipenem (Cayman Chemical, https://www.caymanchem.com), and 10 μL of either 1 mg/mL polymyxin B nonapeptide or 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma-Aldrich, https://www.sigmaaldrich.com), which act as weak or strong permeabilizers, respectively. To start the assay, 25 μL of 1×10^{10} CFU/mL bacterial suspension made by colonies grown overnight on BHI agar (Sigma-Aldrich) is added to each well. To monitor the increase rate, fluorescence measurement is then performed at 37°C at 10-s intervals for 10 min with Ex/Em 450/510 nm in the plate reader (Spectramax M5 plate reader, Molecular Devices, https://www.moleculardevices.com). For each bacterial sample, we performed the reactions in duplicate and averaged the results. We objectively interpreted the fluorescence measurements by using an automated Python script (Appendix), which required a few seconds after assay completion.

We tested FIBA on 76 randomly selected infection isolates from either the Centers for Disease Control and Prevention (*9*) or the American Type Culture Collection (https://www.atcc.org). The MICs of these isolates, if not predetermined, were measured by the 2017 Clinical Laboratory and Standards Institute (https://clsi.org) broth dilution method. Genetic test results for β-lactam resistance were provided with the isolates. Among these, 55
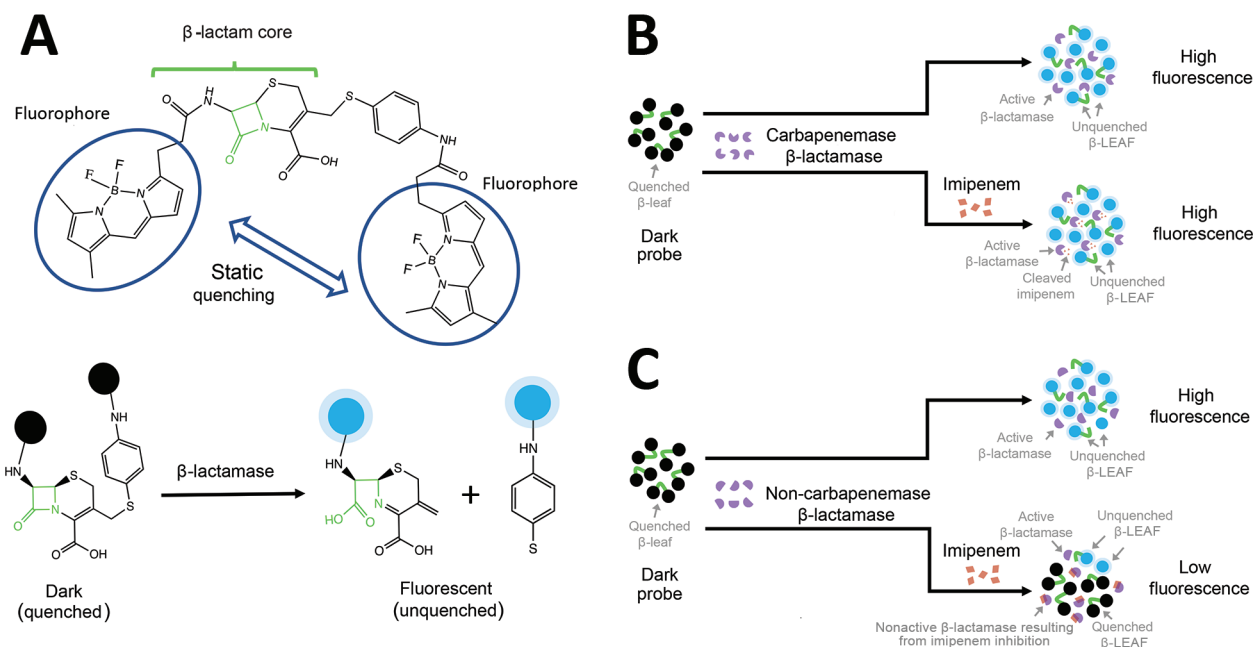
Figure. Schematic illustration of the principle of fluorescence identification of β-lactamase activity. A). The β-lactamase–activated fluorophore probe comprises a cleavable β-lactam core conjugated to 2 fluorophores (circled) that are quenched because of close proximity. This construct was designed to mimic the enzymatic degradation properties of easily cleavable β-lactam antimicrobial drugs. When this probe is attacked by β-lactamase, the probe core is cleaved, leading to the separation of the fluorophores and the recovery of their fluorescent properties (fluorescent state). B) Assay profile for carbapenemase-producing bacteria. C) Assay profile for non–carbapenemase-producing bacteria. Black, quenched fluorophore; blue, unquenched fluorophore turning fluorescent; green, β-lactam core; red, imipenem; purple, β-lactamase. β-LEAF, β-lactamase enzyme–activated fluorophore.

were carbapenemase positive, carrying the major epidemic carbapenemase types including *K. pneumoniae* carbapenemase (n = 20), imipenem-hydrolyzing β-lactamase (n = 2), metallo-β-lactamase (n = 4), New Delhi metallo-β-lactamase (n = 10), oxacillinase (n = 8), *S. marcescens* enzyme (n = 2), São Paulo metallo-β-lactamase (n = 1), Verona integron-encoded metallo-β-lactamase (n = 6), and New Delhi metallo-β-lactamase oxacillinase (n = 2). The other 21 isolates expressed noncarbapenemase β-lactamases, which involved 9 isolates with only ESBL, 3 isolates with both ESBL and porin modification, 6 isolates with only AmpC β-lactamase, and 3 isolates with both ESBL and AmpC β-lactamase. Among these isolates, 3 were carbapenem resistant. The entire panel, which included 28 colistin-resistant strains (MIC ≥4 ug/mL), was classified successfully with FIBA (Appendix Tables 1, 2), resulting in 100% sensitivity (95% CI 94%–100%) and 100% specificity (95% CI 84%–100%).

The primary limitation of this study is the small number of isolates evaluated. However, the breadth of isolates studied here included 8 enzyme types across 16 species, suggesting the generality of the approach.

FIBA can be performed ≈10 times faster than the most rapid carbapenemase test commercially available while maintaining comparable sensitivity and specificity (*6,10*). Its automated analysis improves turnaround time and reduces operator variability. With a reagent cost/assay of ≈US $1, FIBA is close in price to phenotypic tests but substantially faster and less labor intensive. Furthermore, the FIBA paradigm is extensible; by replacing imipenem with other known subtype-dependent inhibitors of carbapenemase (e.g., clavulanic acid, EDTA), rapid carbapenemase subtyping may also be possible. Our study demonstrates that low-cost, rapid assessment of carbapenemase can be performed in a 1-step format suitable for large-scale epidemiologic studies, thereby providing a new tool for infection outbreak control.

About the Author

Dr. Feng is a research fellow at Massachusetts General Hospital and Harvard Medical School, Boston. Her major research interests are molecular mechanisms, epidemiology, and prevention of drug resistance in bacteria.

References

1. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev. 2007;20:440–58. https://doi.org/10.1128/CMR.00001-07
2. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA. Carbapenems: past, present, and future. Antimicrob Agents Chemother. 2011;55:4943–60. https://doi.org/10.1128/AAC.00296-11
3. Tamma PD, Opene BN, Gluck A, Chambers KK, Carroll KC, Simner PJ. Comparison of 11 phenotypic assays for accurate detection of carbapenemase-producing *Enterobacteriaceae*. J Clin Microbiol. 2017;55:1046–55. https://doi.org/10.1128/JCM.02338-16
4. Lasserre C, De Saint Martin L, Cuzon G, Bogaerts P, Lamar E, Glupczynski Y, et al. Efficient detection of carbapenemase activity in *Enterobacteriaceae* by matrix-assisted laser desorption ionization–time of flight mass spectrometry in less than 30 minutes. J Clin Microbiol. 2015;53:2163–71. https://doi.org/10.1128/JCM.03467-14
5. Monteiro J, Widen RH, Pignatari AC, Kubasek C, Silbert S. Rapid detection of carbapenemase genes by multiplex real-time PCR. J Antimicrob Chemother. 2012;67:906–9. https://doi.org/10.1093/jac/dkr563
6. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis. 2012;18:1503–7. https://doi.org/10.3201/eid1809.120355
7. Sallum UW, Zheng X, Verma S, Hasan T. Rapid functional definition of extended spectrum β-lactamase activity in bacterial cultures via competitive inhibition of fluorescent substrate cleavage. Photochem Photobiol. 2010;86:1267–71. https://doi.org/10.1111/j.1751-1097.2010.00801.x
8. Khan S, Sallum UW, Zheng X, Nau GJ, Hasan T. Rapid optical determination of β-lactamase and antibiotic activity. BMC Microbiol. 2014;14:84. https://doi.org/10.1186/1471-2180-14-84
9. CDC and FDA Antibiotic Resistance Isolate Bank [cited 2019 Mar 8]. https://wwwn.cdc.gov/ARIsolateBank
10. Maurer FP, Castelberg C, Quiblier C, Bloemberg GV, Hombach M. Evaluation of carbapenemase screening and confirmation tests with *Enterobacteriaceae* and development of a practical diagnostic algorithm. J Clin Microbiol. 2015;53:95–104. https://doi.org/10.1128/JCM.01692-14

Arthritis Caused by MRSA CC398 in Patient without Animal Contact, Japan

Hidemasa Nakaminami,[1] Yuji Hirai,[1] Hirosuke Nishimura, Shunsuke Takadama, Norihisa Noguchi

Author affiliations: Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan (H. Nakaminami, S. Takadama, N. Noguchi); Tokyo Medical University Hachioji Medical Center, Tokyo (Y. Hirai, H. Nishimura)

Clonal complex 398 methicillin-resistant *Staphylococcus aureus* (MRSA) is a typical lineage of livestock-associated MRSA. We report a case of intractable arthritis of the shoulder joint caused by a multidrug-resistant Panton-Valentine leukocidin–positive livestock-associated MRSA clonal complex 398 sequence type 1232 clone in a patient in Japan who had no animal contact.

In the past decade, methicillin-resistant *Staphylococcus aureus* (MRSA) has been detected in livestock, including swine, poultry, and veal calves (*1,2*). In general, the virulence of animal-derived livestock-associated (LA-MRSA) strains is considered to be lower than that of community-acquired MRSA lineages (*3*). However, LA-MRSA strains can effectively colonize and infect humans, with subsequent transmission in both community and hospital settings. Human colonization with LA-MRSA sequence type (ST) 398 was first recognized among swine farmers in France and the Netherlands in the early 2000s (*4*). According to Larsen et al., clonal complex (CC) 398 MRSA accounted for 21% of MRSA isolated from skin and soft tissue infections in Denmark during 2010–2015 (*5*). However, ST398 MRSA has not been isolated in patients in Japan. We report a case of intractable arthritis of the shoulder joint caused by a multidrug-resistant Panton-Valentine leukocidin (PVL)–positive LA-MRSA CC398 (ST1232) clone in a patient in Japan who had no animal contact.

We performed MRSA identification, staphylococcal cassette chromosome (SCC) *mec* typing, *spa* typing, multilocus sequence typing (MLST), MIC determination, and PCR assays for detecting virulence factors and antimicrobial resistance genes, as described previously (*1,6*). The study protocol was approved by the Tokyo University of Pharmacy and Life Sciences Ethics Committee (approval no. 12–09).

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[1]These authors contributed equally to this article.

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Appendix

Supplementary Methods

The imipenem solution was prepared and used within 30 mins (stored at 4 °C during this time). Samples were physically tested in a blind and random fashion. To develop the subsequent analysis, the data were unblinded to determine the appropriate analysis thresholds. The FIBA limit of detection was $10^{9\text{-}10}$ CFU/ml for this panel.

The automated Python analysis for FIBA test

To quickly determine carbapenemase activity from the fluorescence time course data, a python script was developed to easily analyze the excel spreadsheet files generated by the fluorescence plate reader. The analysis is as follows: To quantify the changes of the β-lactamase activity upon the addition of imipenem, a β-lactamase inhibition index (**BI,** see equation **II**), defined as the ratio of fluorescence increase rate (**R,** see equation **I**) between wells without and with imipenem, was created. **BI** increases with imipenem inhibition (non-carbapenemase β-lactamase behavior), and an isolate with **BI**≤0 is classified as carbapenemase-positive.

Among the samples with **BI**>0 in the presence of 100 μg/ml cell membrane permeabilizer polymyxin B nonapeptide (PMBN), a parallel assay with another permeabilizer, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), was also performed. This is to rule out false negatives that may be caused by insufficient permeabilization due to bacterial polymyxin resistance. Isolates with a **BI**≤0 under the condition of CHAPS were also classified as carbapenemase-positive. All remaining isolates were classified as carbapenemase-negative.

The equations used in Python analysis:

I.

$$R = \sum_{i=1}^{N} v_i$$

where *v* is the fluorescence increase per time step, *i* is the time index of the sorted *v* (i.e., v_1 is the largest fluorescent difference, v_2 is the second largest fluorescent difference, etc.) and *N* is the number of *v* to be summed. *v* was determined from a 50 second running average of the fluorescent time course, and *N* was set to 45.

II.

$$BI = \left(\sum_{i=1}^{N} v_i^{0\,\mu M} \middle/ \sum_{i=1}^{N} v_i^{10\,\mu M} \right) - BI_0$$

where v_i^C is the fluorescence increase per time step at imipenem concentration C , and BI_0, designated as the **BI** cut-off value for the enzyme inhibition, is 1.25.

Appendix Table 1. Carbapenemase-producing isolates subjected to the FIBA* test

Carbapenemase category					Carbapenem susceptibility‡				FIBA test
Ambler class	Type	Subtype	Species	No.†	Imi	Mer	Ert	Dor	result§
A	KPC	KPC-2	*C.freundii*	1	16	> 8	> 8	> 8	+
			E.cloacae	1	8	8	> 8	4	+
			K.pneumoniae	1	> 8	16	≤ 0.25	> 8	+
			M.morganii	1	8	4	8	4	+
			P.mirabilis	1	16	0.5	1	4	+
		KPC-3	*E.cloacae*	2	≥8	4-8	0.5	1- > 8	+
			E.coli	2	4-8	4	0.5 - 8	≤ 0.25 - 4	+
			K.ascorbata	1	4	8	8	4	+
			K.oxytoca	1	4	1	0.5	0.5	+
			K.ozaenae	1	> 8	> 64	0.5	> 8	+
			K.pneumoniae	3	> 8	> 8	≥ 4	> 8	+
			R.ornithinolytica	1	4	1	1	2	+
		KPC-4	*E.cloacae*	1	0.5	1	0.5	> 8	+
		KPC-5	*P.aeruginosa*	1	> 8	> 64	1	> 8	+
		KPC-6	*E.cloacae*	1	4	4	0.5	8	+
			P.mirabilis	1	16	2	2	2	+
	SME	SME-3	*S.marcescens*	2	> 8	> 64	4	≤ 0.25- 0.5	+
	NMC-A	NMC-A	*E.cloacae*	2	≥ 32	> 8	> 8	> 8	+
B	NDM	NDM-1	*E.coli*	1	8	> 16	> 8	> 8	+
			K.pneumoniae	2	> 8	> 8	1 - > 8	> 8	+
			M.morganii	1	2	8	4	> 8	+
			P.mirabilis	1	32	4	4	> 8	+
			P.rettgeri	1	8	32	4	≤ 0.25	+
			S.senftenberg	1	4	8	> 8	8	+
			A.baumannii	1	> 8	64	1	> 8	+
			Citrobacter spp.	1	16	> 8	> 8	> 8	+
			E.cloacae	1	4	32	≤ 0.25	> 8	+
		NDM-1/OXA-64	*A.baumannii*	1	> 8	64	0.5	> 8	+
		NDM-6	*E.coli*	1	16	> 8	> 8	> 8	+
	VIM	VIM-1	*E.cloacae*	1	4	2	2	4	+
			K.pneumoniae	1	4	4	1	4	+
		VIM-2	*P.aeruginosa*	2	> 64	> 8	> 8	> 8	+
		VIM-27	*K.pneumoniae*	1	64	> 8	> 8	> 8	+
		VIM-4	*P.aeruginosa*	1	> 64	> 8	> 8	4	+
	IMP	IMP-1	*P.aeruginosa*	1	> 64	> 8	> 8	> 8	+
		IMP-14	*P.aeruginosa*	1	64	> 8	> 8	> 8	+
		IMP-4	*K.pneumoniae*	2	1 - 4	2 - 4	2 - 4	4 - 8	+

Carbapenemase category			Species	No.†	Carbapenem susceptibility‡				FIBA test result§
Ambler class	Type	Subtype			Imi	Mer	Ert	Dor	
	SPM	SPM-1	*P.aeruginosa*	1	> 64	> 8	> 8	> 8	+
D	OXA	OXA-48	*E.aerogenes*	1	4	2	2	2	+
			K.pneumoniae	1	4	8	> 8	8	+
		OXA-58/100	*A.baumannii*	2	16 - 32	> 8	> 8	8	+
		OXA-66/72	*A.baumannii*	1	> 64	> 8	> 8	> 8	+
		OXA-181	*K.ozaenae*	1	4	4	> 8	4	+
			K.pneumoniae	1	2	4	> 8	4	+
		OXA-232	*K.pneumoniae*	1	4	> 8	> 8	> 8	+

*FIBA, Fluorescence identification of β-Lactamase activity.
†No., number of isolates tested.
‡MIC of the tested isolates for doripenem (Dor), ertapenem(Ert), imipenem(Imi) and meropenem(Mer).
§FIBA test result: -, negative; +, positive. All the results shown here were based on the average of two independent replicates. With the permeabilizer PMBN, there are 4 out of 57 (7%) isolates labeled as false negatives which are subsequently found positive with the permeabilizer CHAPS.

Appendix Table 2. Non-carbapenemase-producing isolates subjected to the FIBA[*] test

| β-Lactamase category		Species	No.†	Carbapenem susceptibility‡				FIBA test result§
Type	Subtype			Imi	Mer	Ert	Dor	
ESBL	CTX-M-14, TEM-1B	*E.coli*	1	≤ 0.5	≤ 0.12	1	≤ 0.12	-
	CTX-M-15, SHV-1, TEM-1B, OXA-1/10¶	*K.pneumoniae*	1	1	2	> 8	2	-
	CTX-M-2, SHV-83, TEM-1A, OXA-9/10¶	*K.pneumoniae*	1	8	> 8	> 8	> 8	-
	SHV-3	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	SHV-4	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	SHV-12	*K.pneumoniae*	1	≤ 0.5	2	> 8	1	-
	SHV-12¶	*K.pneumoniae*	1	≤ 0.5	≤ 0.12	0.25	≤ 0.12	-
	TEM-3	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	TEM-10	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	TEM-12	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	TEM-26	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
	TEM-52B	*E.coli*	1	≤ 0.5	0.25	1	0.25	-
AmpC	ACT-7	*E.cloacae*	1	≤ 0.5	≤ 0.12	0.25	≤ 0.12	-
	ACT-15	*E.cloacae*	1	≤ 0.5	≤ 0.12	1	≤ 0.12	-
	CMY-2	*E.coli*	2	≤ 0.5	≤ 0.12 - 1	≤ 0.12 - 2	≤ 0.12	-
	cAmpC	*E.aerogenes*	1	≤ 0.5	≤ 0.12	1	≤ 0.12	-
	cAmpC	*E.cloacae*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-
ESBL& AmpC	CTX-M14; DHA-1, SHV-11, TEM-1B¶	*K.pneumoniae*	1	16	8	> 8	8	-
	cAmpC, TEM-1B	*E.cloacae*	1	≤ 0.5	≤ 0.12	1	≤ 0.12	-
	CMY-2, TEM-1B	*E.coli*	1	≤ 0.5	≤ 0.12	≤ 0.12	≤ 0.12	-

*FIBA, fluorescence identification of β-Lactamase activity.
†No., number of isolates tested.
‡MIC of the tested isolates for doripenem (Dor), ertapenem(Ert), imipenem(Imi) and meropenem(Mer).
§FIBA test result: -, negative; +, positive.
¶Porin modifications were present together with β-lactamase. All the results shown here were based on the average of two independent replicates.
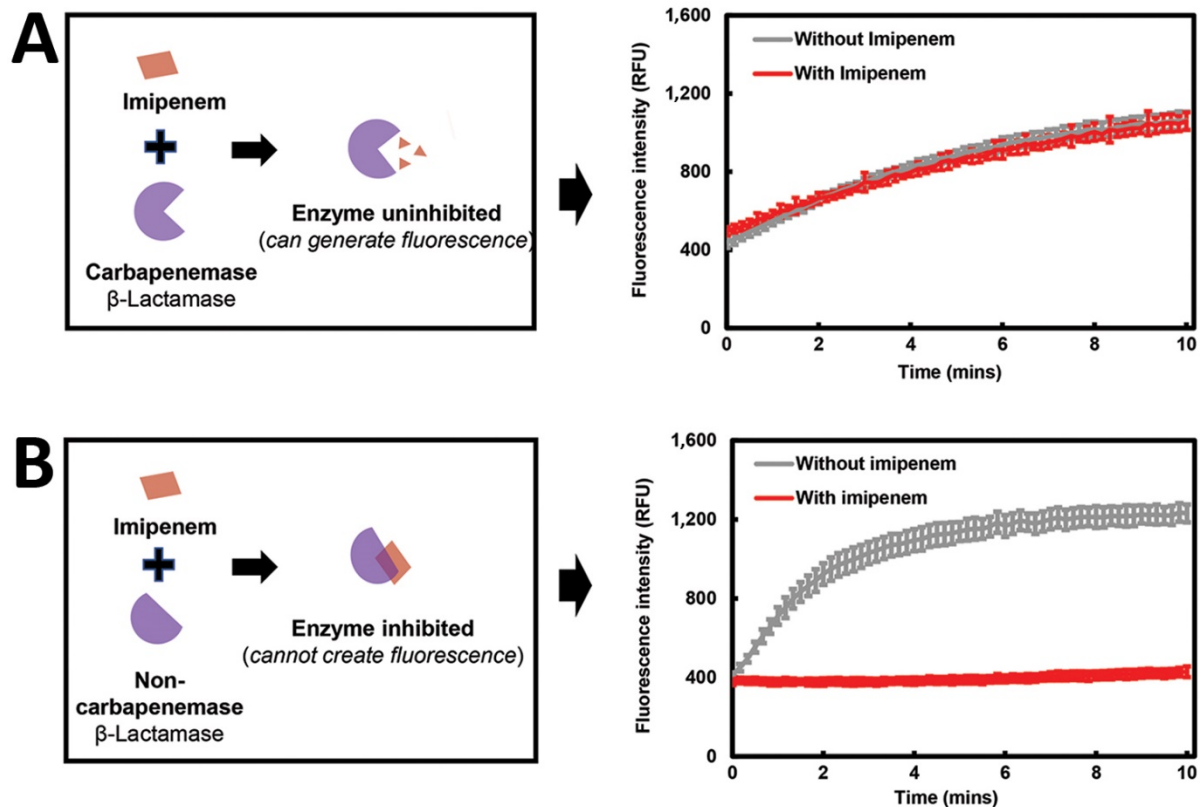
Appendix Figure 1. The detection of bacterial carbapenemase production by the fluorescence identification of β-lactamase activity. A) Carbapenemase producing isolates cleave β-lactamase enzyme-activated fluorophore irrespective of imipenem addition, as exemplified here by the strain # 0147 from the CDC isolate bank. B) Non-carbapenemase β-lactamases are unable to cleave β-lactamase enzyme-activated fluorophore when inhibited by imipenem, as shown here by the isolate # 0065 from the CDC isolate bank.
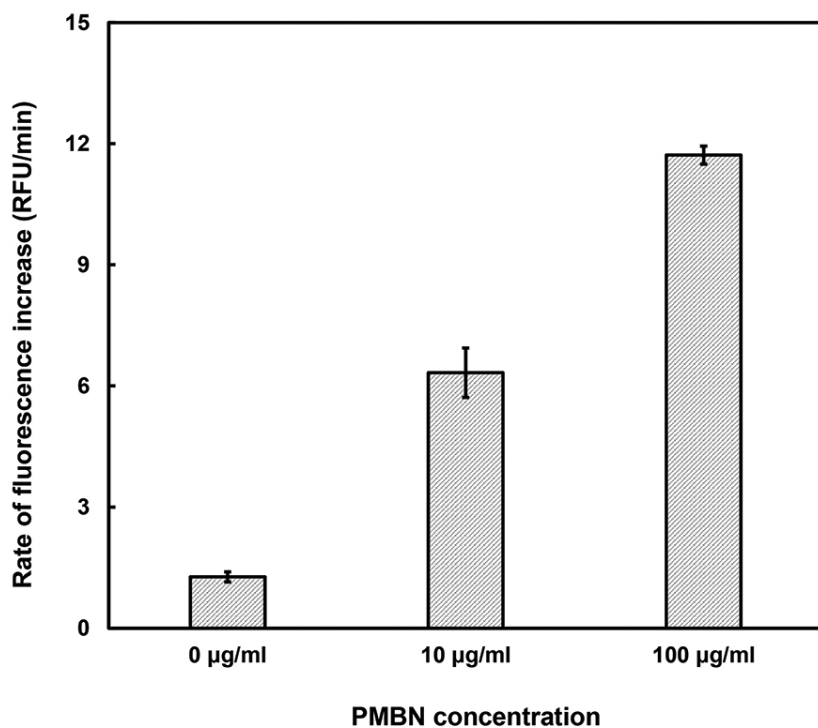
Appendix Figure 2. The rate of fluorescence increase in FIBA* increases with the addition of PMBN[†].
*FIBA, fluorescence identification of β-lactamase activity; [†]PMBN, polymyxin B nonapeptide; The strain used here as an illustration is a β-lactamase producing strain from ATCC (*Escherichia coli*, ATCC® BAA-196™).